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# **Reductive activation of 5-fluorodeoxyuridine prodrug possessing azide methyl group by hypoxic X-irradiation**

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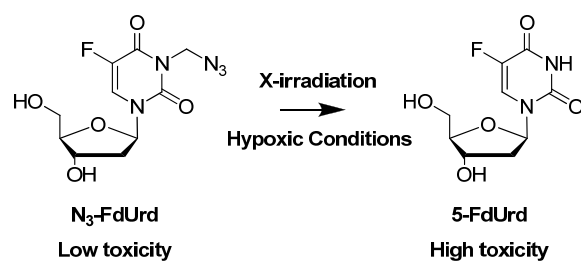
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## Graphical Abstract



## Abstract

We prepared a 5-fluorodeoxyuridine (5-FdUrd) derivative possessing azide methyl group ( $N_3$ -FdUrd) as a novel radiation-activated prodrug. The parent antitumor agent, 5-FdUrd, was released efficiently from  $N_3$ -FdUrd by hypoxic X-irradiation. On the other hand, the activation of  $N_3$ -FdUrd was suppressed upon X-irradiation under aerobic conditions. A biological assay using A549 cells revealed that the cytotoxicity of  $N_3$ -FdUrd was significantly enhanced by hypoxic X-irradiation.

Prodrugs that are activated by an external stimulus have been attracting much attention.

These prodrugs themselves are nontoxic, but exposure to triggers such as photoirradiation,<sup>1</sup> change in pH<sup>2</sup> or enzymatic reactions<sup>3</sup> bring about changes in the chemical structure of the prodrugs to enable their inherent medical capability.<sup>4</sup> Because of the ease of controlling their functions, stimuli-sensitive prodrugs are prospective high-performing medical agents with mild side effects.

One of the new strategies employed in this area is the utilization of X-irradiation<sup>5</sup> as an external trigger to activate prodrugs. Since chemical reactions triggered by radiation can be controlled spatially and temporary, such prodrugs can be converted to their active forms with exact control of the area, time, and dosage. Recently, we have developed several prodrugs that can be activated by X-irradiation.<sup>6</sup> We prepared prodrugs of the antitumor agents 5-fluorouracil, 5-fluorodeoxyuridine (5-FdUrd), and cytarabine by incorporation of a 2-oxoalkyl group or an indolequinone structure, which are removable by X-ray treatment under hypoxic conditions. A reaction mechanism has been proposed: these substituents undergo reductive activation by reducing species generated by the radiolysis of water<sup>7</sup> to release their parent drug. These prodrugs showed cytotoxicity

towards hypoxic tumor cells upon X-irradiation, while they were nontoxic against aerobic cells, even upon X-irradiation.

Herein, we discuss the design of a novel radiation-activated prodrug of 5-FdUrd possessing azide methyl group ( $N_3$ -FdUrd), which is known to be a removable substituent under reducing conditions.<sup>8</sup> We expected that  $N_3$ -FdUrd would be activated by reducing species generated from radiolysis of water to form amination 1 (Figure 1), which would be hydrolyzed in aqueous solution to give 5-FdUrd. We synthesized the  $N_3$ -FdUrd and characterized its radiolytic reduction behavior. The radiation-dependent cytotoxicity of  $N_3$ -FdUrd using living cells was also assessed.

The strategy for preparation of  $N_3$ -FdUrd is summarized in Scheme 1. Diacetyl-5-fluorodeoxyuridine 2 was coupled with chloromethyl methyl sulfide, and then converted to the azide compound 4.<sup>9</sup> Hydrolysis of 4 under basic conditions furnished the desired  $N_3$ -FdUrd.<sup>10</sup>  $N_3$ -FdUrd had good water solubility as well as 5-FdUrd.

(Figure 1)

(Scheme 1)

Initially, we performed the radiolytic reduction of N<sub>3</sub>-FdUrd and monitored the reaction using reversed-phase HPLC.<sup>11</sup> We conducted the radiolysis in argon-purged aqueous solutions containing 2-methyl-2-propanol as a scavenger for the oxidizing hydroxyl radicals (OH<sup>•</sup>). Under these conditions, hydrated electrons (e<sub>aq</sub><sup>-</sup>) and hydrogen atoms (H<sup>•</sup>) are generated as the major reducing species from the radiolysis of water. As shown in Figure 2, a single new signal appeared during radiolysis under hypoxic conditions, while the starting material, N<sub>3</sub>-FdUrd, disappeared. The product was identified as the parent 5-FdUrd, according to the overlapped injection of authentic samples in the HPLC analysis. The G values were estimated to be 159 nmol/J for the formation of the corresponding 5-FdUrd and 336 nmol/J for the decomposition of N<sub>3</sub>-FdUrd; thus, 47% of the decomposed N<sub>3</sub>-FdUrd was converted to 5-FdUrd upon hypoxic X-irradiation. To verify the reaction mechanism in detail, we next conducted a similar radiolytic reduction of N<sub>3</sub>-FdUrd under aerobic conditions. In contrast to the efficient activation of hypoxic irradiation, both formation of 5-FdUrd and decomposition of N<sub>3</sub>-FdUrd were

markedly suppressed, as shown in Figure 2b. The G values were 50 nmol / J for the formation of 5-FdUrd and 118 nmol / J for the decomposition of N<sub>3</sub>-FdUrd. It has been well-characterized that molecular oxygen captures both  $e_{aq}^-$  and  $H^\bullet$ .<sup>12</sup> Thus, it is most likely that  $e_{aq}^-$  and  $H^\bullet$  are key reactive species for the activation of N<sub>3</sub>-FdUrd and the formation of 5-FdUrd.

(Figure 2)

We then assessed the cytotoxicity of N<sub>3</sub>-FdUrd and 5-FdUrd towards A549 cells (human lung adenocarcinoma). A cell viability assay was employed to test the cytotoxicity in vitro. The IC<sub>50</sub> value was defined as the concentration required to reduce the viability of the cells by 50%, and was estimated on exposure to N<sub>3</sub>-FdUrd and 5-FdUrd.<sup>13</sup> Figure 3 shows the survival curve of the A549 cells incubated in the presence of drugs at various concentrations in air containing 5% CO<sub>2</sub> for 72 h at 37 °C. The IC<sub>50</sub> values were 1.47 and 0.047 μM for N<sub>3</sub>-FdUrd and 5-FdUrd, respectively, indicating that incorporation of an azide methyl group into 5-FdUrd at the N(3)-position is responsible for the decrease



in the cytotoxic effect of 5-FdUrd.

(Figure 3)

The properties of N<sub>3</sub>-FdUrd described above prompted us to further investigate the effect of X-irradiation on the cytotoxicity (Figure 4A). An aqueous solution of N<sub>3</sub>-FdUrd was X-irradiated under hypoxic or aerobic conditions, and then the resulting solution was administered to the A549 cells.<sup>14</sup> In accordance with the suppression of the cytotoxic effects mentioned above, administration of 200 nM N<sub>3</sub>-FdUrd did not bring about a cytotoxic action. We also confirmed that the X-irradiated N<sub>3</sub>-FdUrd under aerobic conditions did not show any cytotoxic effect, consistent with the results that radiolytic activation is suppressed under aerobic conditions. It is striking that the cell viability was significantly reduced in the presence of hypoxically irradiated N<sub>3</sub>-FdUrd. These results strongly indicate that 5-FdUrd released from N<sub>3</sub>-FdUrd upon hypoxic irradiation showed strong cytotoxicity towards A549 cells.

(Figure 4)

In light of the effect of X-irradiation, further attempts were made to verify the function of N<sub>3</sub>-FdUrd as a radiation-activated prodrug. We exposed A549 cells to X-rays under hypoxic conditions in the presence or absence of N<sub>3</sub>-FdUrd and characterized the radiation-dependent cytotoxic effect of N<sub>3</sub>-FdUrd.<sup>15</sup> As shown in Figure 4B, the cells were viable even in the presence of 100 nM N<sub>3</sub>-FdUrd without X-irradiation. Although the hypoxic cells showed high viability even upon X-irradiation owing to their resistibility, radiation in the presence of N<sub>3</sub>-FdUrd significantly enhanced the radiation sensitivity of the A549 cells, resulting in low cell viability. Thus, N<sub>3</sub>-FdUrd released toxic 5-FdUrd via radiolytic reduction in hypoxic cells, which led to an enhanced cytotoxicity.

In summary, we have synthesized a 5-FdUrd derivative possessing azide methyl group at the N(3)-position (N<sub>3</sub>-FdUrd) and characterized its properties upon X-irradiation. N<sub>3</sub>-FdUrd was activated to release 5-FdUrd in a hypoxia-selective manner via reduction by H<sup>•</sup> and e<sub>aq</sub><sup>-</sup>, which were generated by the radiolysis of water. A cytotoxicity assay

using A549 cells revealed that N<sub>3</sub>-FdUrd showed a lower toxicity. On the other hand, X-irradiation resulted in a formation of 5-FdUrd in hypoxic cells, leading to the enhancement of the radiation sensitivity of A549 cells. Thus, N<sub>3</sub>-FdUrd is a promising radiation-activated prodrug with lesser side effects. Further in vivo experiments on N<sub>3</sub>-FdUrd, focusing on practical use, are in progress.

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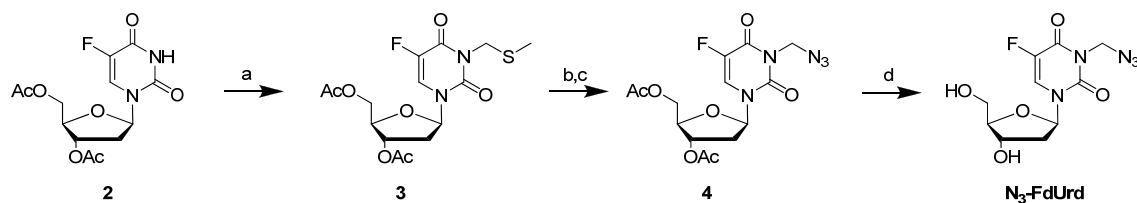
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- (10) ***N*<sub>3</sub>-FdUrd**: Colorless oil; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  2.12–2.18 (2H), 3.57 (ddd, *J* = 3.42, 4.88, 12.20 Hz, 1H), 3.64 (ddd, *J* = 3.42, 8.78, 12.20 Hz, 1H), 3.81 (dd, *J* = 3.41, 6.80 Hz, 1H), 4.24 (m, 1H), 5.15–5.21 (1H). 5.22 (s, 1H), 5.27 (d, *J* = 4.40, 1H), 6.15 (dd, *J* = 6.30, 6.30 Hz, 1H), 8.37 (d, *J* = 6.83 Hz, 1H); <sup>13</sup>C NMR (67.8 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  55.8, 60.7, 69.8, 85.6, 87.7, 124.5 (d, *J* = 34.4 Hz), 138.1, 140.9, 148.8, 156.0; FABMS: *m/e* 302 [(M+H)<sup>+</sup>]; HRMS calcd for C<sub>10</sub>H<sub>13</sub>N<sub>5</sub>O<sub>5</sub>F [(M+H)<sup>+</sup>] 302.0901, found 302.0900.
- (11) Since the large amount of prodrug and products was necessary to monitor the reaction by HPLC, we conducted the experiment using 300  $\mu$ M prodrugs and high dose of X-irradiation (up to 960 Gy).
- (12) Reducing hydrated electrons and hydrogen atoms are scavenged dissolved O<sub>2</sub> (*k* =  $1.9 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$  for hydrated electrons, *k* =  $2.1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$  for hydrogen atoms). See ref. 7.
- (13) **Assessment of cytotoxicity toward A549 cells**: A549 cells were cultured in

Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were seeded into 96-well plates (2000 cells/well) and cultured at 37 °C in a well-humidified incubator with 5% CO<sub>2</sub> and 95% air (aerobic condition) for 24 hours. The cells were then incubated with the various concentrations of **5-FdUrd** or **N<sub>3</sub>-FdUrd** under aerobic conditions for 72 hours, and added with 10 µL of Cell Counting kit-8 (WST-8, DOJINDO, Japan). The plates were further incubated at 37 °C for 30 min and the cell viability assay was performed using Microplate spectrophotometer (BIO-RAD).

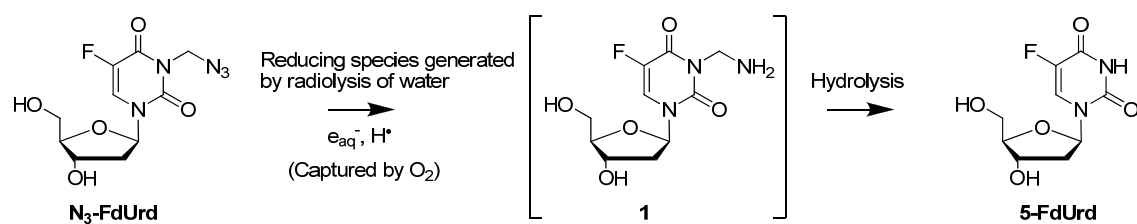
- (14) **Radiation-induced cytotoxicity of pre-irradiated N<sub>3</sub>-FdUrd:** A549 cells were seeded into 96-well plates (2000 cells / well) and incubated at 37 °C for 24 hours under aerobic conditions. An aqueous solution of N<sub>3</sub>-FdUrd (0 or 2 µM) was irradiated under aerobic or hypoxic conditions and then added to the cells. Similar to above, we performed cell viability assay to measure an antiproliferative activity (See ref. 13).
- (15) **Radiation-induced cytotoxicity of N<sub>3</sub>-FdUrd (X-irradiation to cells containing N<sub>3</sub>-FdUrd):** For the hypoxic treatment (< 0.02% of oxygen), the

cells were treated in a Anaeron Pack System (Mitsubishi Gas Chemical Company Inc., Japan) equipped with AnaeroPouch-Anaero (Mitsubishi Gas Chemical Co. Ltd, Japan). The 96-well plates (2000 cells/well) kept under hypoxic conditions using Anaeron Pack System were X-irradiated (0 and 6 Gy) and incubated for 72 hours under aerobic conditions. The cell viability assay was performed as described above (See ref. 13).

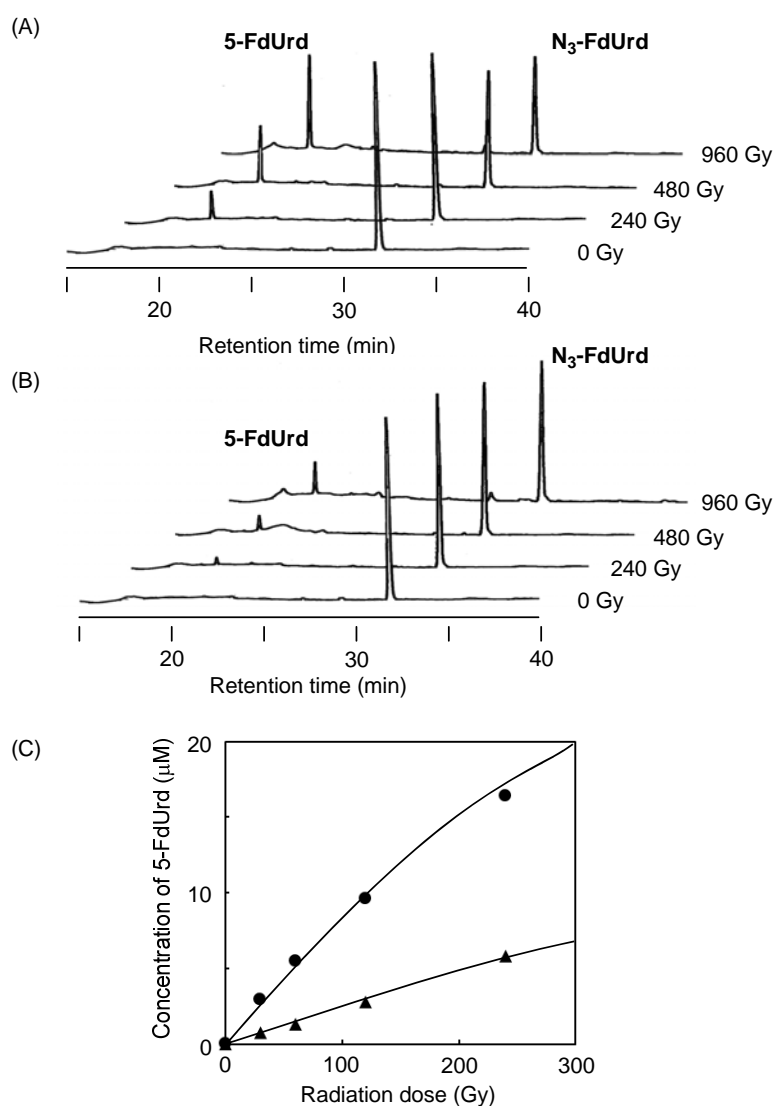




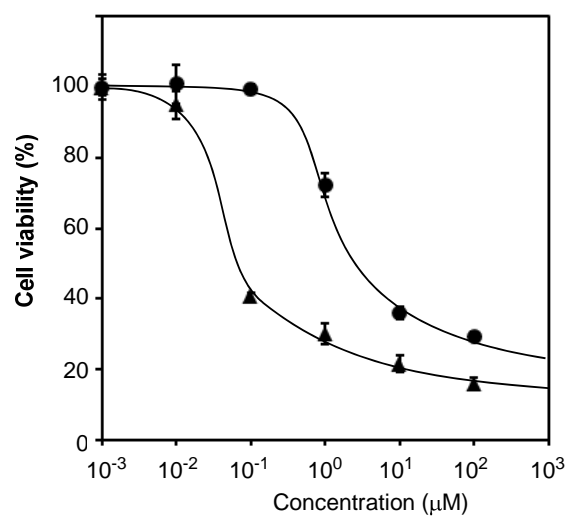
**Scheme 1.** Reagents and conditions: (a) Chloromethylmethylsulfide, NaH, DMF, 2 h, r.t., 78%; (b) NCS, TMSCl, 2 h, r.t., CH<sub>2</sub>Cl<sub>2</sub>; (c) NaN<sub>3</sub>, DMF, 48 h, r.t., 17% (2 steps); (d) NaOH, MeOH, H<sub>2</sub>O, 3 h, 0 °C, quant.



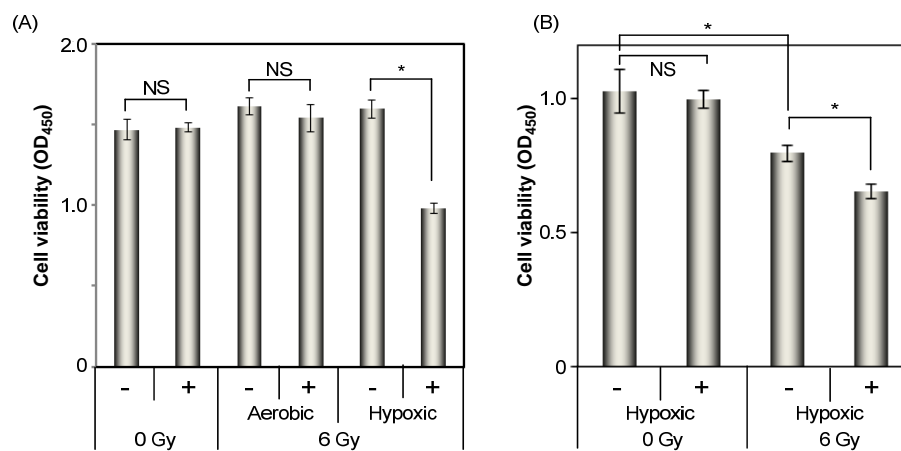
**Figure 1.** Plausible reaction mechanism for the formation of 5-FdUrd upon hypoxic X-irradiation.



**Figure 2.** (A,B) HPLC profiles for the reductive activation of N<sub>3</sub>-FdUrd (300 μM) by X-irradiation (0, 240, 480 and 960 Gy). The radiolysis of aqueous solution containing 15 mM 2-methyl-2-propanol was conducted under hypoxic (A) or aerobic (B) conditions. (C) Release of 5-FdUrd from N<sub>3</sub>-FdUrd (300 μM) in the hypoxic (circle) or aerobic (triangle) radiolysis.



**Figure 3.** Cytotoxicity of N<sub>3</sub>-FdUrd (circle) and 5-FdUrd (triangle) against A549 tumor cells. A549 cells were incubated with indicated concentrations of N<sub>3</sub>-FdUrd or 5-FdUrd under aerobic conditions for 72 h. The cell viability was calculated by means of cell counting kit-8 (WST-8). Results are shown with the mean  $\pm$  S.D. (n = 4).



**Figure 4.** Radiation-induced cytotoxicity of N<sub>3</sub>-FdUrd against A549 cells. (A) Aqueous solutions of N<sub>3</sub>-FdUrd (0 nM: - or 200 nM: +) was X-irradiated (6 Gy) under aerobic or hypoxic conditions, and then administered to cells. (B) A549 cells were cultured in the presence or absence of 100 nM N<sub>3</sub>-FdUrd, and treated with X-ray (6 Gy) under hypoxic conditions. Results are shown with the mean  $\pm$  S.D. (n = 3 for (A), n = 4 for (B))(\*P < 0.05; NS: not significant).